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CERTIFICATION OF FACSIMILE TRANSMISSION

I hereby certify that the following documents in re Application of Williem F. WOLKERS et al., Application No. 10/802,099, filed March 16, 2004 for THERAPEUTIC PLATELETS AND METHODS are being facsimile transmitted to the Patent and Trademark Office on the date shown below.

Documents Attached

1. Amendment (14 pages); and Lin et al Pages 9121-9126(attached)
2. PTO/SB/22 - Petition for Extension of Time (1 page submitted in duplicate)
3. PTO/SB/31- Notice of Appeal - 1 page submitted in duplicate)

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is secretion-dependent, but agonist- and aggregation-independent.

Antigen Specificity—The specificity of the antibody for a platelet antigen was examined using the Western blot method. For purposes of comparison, platelet proteins from thrombin-activated platelets and resting platelets were solubilized in SDS and analyzed. As shown in Fig. 5, the KC4 antibody bound to a single band in the solubilized thrombin-activated platelets and resting platelets. This band migrated with an apparent molecular weight of 139,000. Platelets, surface-labeled with ^{125}I using the lactoperoxidase method, were run for comparison. The characteristic band pattern of the ^{125}I-labeled platelets showed GPIIb, GPIIa, and GPIII (25). The protein antigen of the KC4 antibody migrated between glycoproteins IIb and IIa. Red blood cells, neutrophils, monocytes, lymphocytes, GM4672 (a lymphocytoid cell line), and Alexander PLC/PRF/5 (a human hepatoma cell line) were solubilized in SDS and their proteins similarly examined for binding to the KC4 antibody using the Western blot method. None of these cells contained proteins which bound to this antibody.

Purification of the KC4 Antigen—The KC4 antigen was purified from crude platelet membranes by affinity chromatography. The proteins were extracted from the membranes using Triton X-100, and these proteins applied to an affinity column containing the KC4 antibody covalently coupled to agarose. The material applied to the column was heterogeneous (Fig. 6) and most of these proteins failed to bind to the KC4 antibody-agarose. The bound protein, eluted with diethylamine, migrated as a major diffuse band on SDS gels upon electrophoresis in nonreducing conditions (Fig. 6). Several minor high molecular weight contaminants were also observed. The dominant protein band corresponded to an apparent molecular weight of 140,000. The character of this band was unchanged in the presence of Ca^{2+} or EDTA. In SDS gels run under reducing conditions, the purified KC4 protein migrated as a single narrow band also with a molecular weight of 140,000. These results indicate that the KC4 protein is composed of a single polypeptide chain.

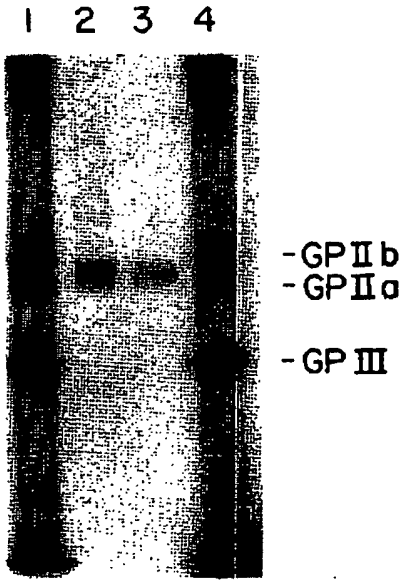

FIG. 5. **Immunoblotting of platelet proteins with the KC4 monoclonal antibody.** SDS extracts of resting and thrombin-activated platelets were prepared and subjected to electrophoresis in nonreduced SDS gels. ^{125}I-surface-labeled platelet proteins were run as standards. After electrophoretic transfer and blotting with ^{125}I-labeled KC4 antibody, the blot was developed by autoradiography. *Lanes 1* and *4*, ^{125}I-labeled platelet proteins; *Lane 2*, solubilized unlabeled resting platelets; *Lane 3*, solubilized unlabeled thrombin-activated platelets. The proteins were blotted with ^{125}I-labeled KC4 antibody. Glycoproteins IIb, IIa, and III are identified, for comparison.

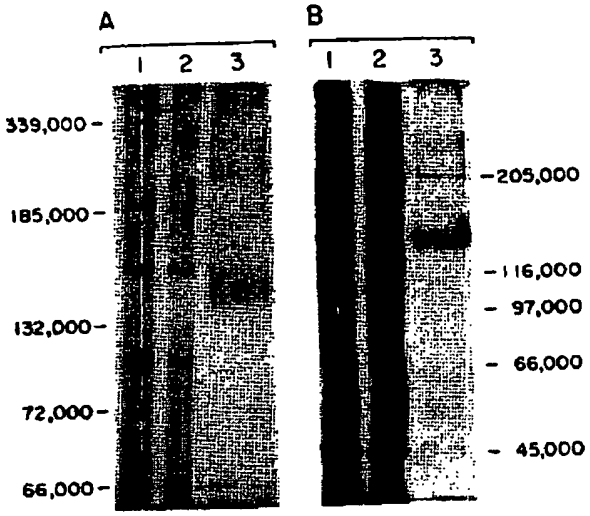

FIG. 6. **SDS gel electrophoresis of immunoaffinity-purified KC4 antigen.** Platelet membrane proteins were extracted into Triton X-100, and the proteins applied to a KC4 antibody-agarose column. The bound protein was eluted with diethylamine and analyzed by SDS gel electrophoresis. *Lane 1*, solubilized platelet membrane proteins; *Lane 2*, solubilized platelet membrane proteins which did not bind to KC4 antibody-agarose; *Lane 3*, bound fraction of solubilized platelet membrane proteins eluted with diethylamine. *A*, nonreduced; *B*, reduced. The migration of proteins of known molecular weight is indicated. Proteins were visualized with silver stain.

DISCUSSION

Like the zymogens of the blood-clotting enzymes and the pro-cofactors which exist in the blood in a precursor form, platelets circulate in the blood as inert cells *vis à vis* their function in coagulation. Upon activation of blood coagulation, platelets undergo a metamorphosis which can be observed biochemically and morphologically. Despite considerable advances in the description of protein components of the platelet membrane (36), incomplete information is available concerning the exposure or expression of receptors and enzyme activities which are unique to the activated platelet membrane surface. To approach this problem, we have prepared monoclonal antibodies specific for activated platelets and characterized the antigen against which one of these antibodies is directed.

The KC4 clone was selected from among a large number of clones producing monoclonal anti-platelet antibodies on the basis of its ability to produce antibody which bound to activated platelets, but not to resting platelets. Because of the difficulty in getting reproducible numbers of platelets bound to the solid phase for the ELISA, we reserved this assay for screening purposes and relied on a solution phase radioimmunoassay to quantitate the reactivity of the KC4 antibodies with resting and activated platelets. Using unfixed gel-filtered platelets, we determined that the KC4 antibody bound to

thrombin-activated platelets with an average binding constant, K_D, of 7.2 nM. These antibodies bound less tightly to resting platelets, 100-fold more antibody being required with resting platelets to give binding comparable to that observed with the thrombin-activated platelets. On the basis of these experiments, it is not possible to distinguish between a pool of resting platelets which is contaminated with 1% activated platelets, platelets which allow partial entrance of antibody into the cell, or the partial expression of the KC4 antigen on the resting platelet surface to which the monoclonal antibody binds with lower affinity. Considerable efforts were taken to maintain the platelets in their resting state, including the use of adenosine and acetylsalicylate. However, KC4 antibody, at high concentration, bound to the resting platelets. Nonetheless, these antibodies demonstrated a marked preference for the activated platelet.

The accumulated data suggest that this is a platelet membrane protein that has not been previously purified. This conclusion is based upon the molecular weight of the protein, the number of KC4 antigens per platelet, and the distribution of the protein in resting and activated platelets. The protein migrated as a single band in SDS gels and yielded an apparent molecular weight of 140,000 under nonreducing and reducing conditions. This band reproducibly migrated between glycoprotein IIb and glycoprotein IIa. Approximately 13,000 KC4 antigens per platelet were observed. This value differs from the estimated number of fibrinogen receptors (40,000 per platelet), a structure attributed to glycoproteins IIb-III (8). However, the recent report of McEver and Martin (37) indicates that their monoclonal antibody specific for thrombin-activated platelets recognizes about 9,000 sites per platelet, a value similar to our own. Further, the platelet antigen that they describe has a similar apparent molecular weight to the KC4 antigen. These results suggest that both the KC4 antibody and the antibody reported by McEver *et al.* may be directed against the same platelet antigen. A recent report by Gogstad *et al.* (38) describes a protein antigen, G18, present in the α-granule membranes but not in the plasma membrane of resting platelets. With a molecular weight of 130,000–135,000 in reduced and unreduced gels, this protein may be related to the KC4 antigen.

The expression of the KC4 protein on the platelet surface was independent of platelet aggregation or the agonist used to activate the platelets. Rather, expression was secretion-dependent, with the extent of KC4 protein expression directly correlated to the extent of platelet secretion. Acetylsalicylate, which inhibits secretion, also inhibited expression of this protein. However, this protein is distinct from other proteins secreted from the α-granules in that thrombospondin, platelet factor four, and β-thromboglobulin are either released from the platelet into the surrounding environment or bind to the platelet plasma membrane through the action of calcium. KC4 antigen remains associated with the isolated platelet membrane. EDTA, solutions of high ionic strength, or solutions of low or high pH failed to dissociate the KC4 antigen from the platelet membrane. These results suggest that the KC4 antigen is an integral membrane protein in the activated platelet. Since KC4 antigen can be identified in solubilized resting platelets by the Western blot technique and can be isolated from a crude membrane fraction containing granule and plasma membranes, it may be a component of a granule membrane. A recent report of a monoclonal antibody, specific for activated platelets, that binds a subpopulation of internal granule membranes in platelets and macrophages is compatible with this hypothesis (39). However, the relation between the KC4 antibody and this antibody remains uncertain.

In summary, we have used an immunologic approach to identify a structure on the postsecretion plasma membrane surface of activated platelets. This platelet-specific protein is composed of a single chain with a molecular weight of about 140,000. Approximately 13,000 sites are expressed on the surface of normal platelets. This protein is an integral membrane protein of the activated plasma membrane. The complete characterization of this protein and the determination of its function should increase our understanding of platelet function and the role of the platelet membrane in hemostasis.

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